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Exogenous FGF-4 Can Suppress Anterior Development in the Mouse Embryo during Neurulation and Early Organogenesis

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Members of the fibroblast growth factor (FGF) family of peptide growth factors are widely expressed in the germ layer derivatives during gastrulation and early organogenesis of the mouse. We have investigated the effect of administering recombinant FGF-4 in the late-primitive streak stage embryo to test if the patterning of the body plan may be influenced by this growth factor. Shortly after FGF treatment the embryonic tissues up-regulated the expression of *Brachyury* and the RTK signaling regulator *Spry2,* suggesting that FGF signaling was activated as an immediate response to exogenous FGF. Concomitantly, *Hesx1* expression was suppressed in the prospective anterior region of the embryo. After 24 h of *in vitro* development, embryos displayed a dosage-related suppression of forebrain morphogenesis, disruption of the midbrain–hindbrain partition, and inhibition of the differentiation of the embryonic mesoderm. Overall, development of the anterior–posterior axis in the late gastrula is sensitive to the delivery of exogenous FGF-4. The early response associated with the expression of *Spry2* suggests that the later phenotype observed could be primarily related to an inhibition of the FGF signaling pathway. © 2000 Academic Press

Key Words: **mouse embryo; FGF-4; *Spry2;* neural axis; anterior–posterior patterning.**

INTRODUCTION

The molecular mechanism underpinning the development of correct anterior–posterior neural characteristics has been the subject of much fascination. How this regionalization of the neural axis is brought about remains largely undefined although several candidate posteriorizing factors have been identified including the fibroblast growth factor (FGF) family of peptide growth factors (Sasai and De Robertis, 1997). Studies of this expanding family of molecules have revealed a complicated ligand–receptor signaling network involving a hierarchy of interactions between multiple functional isoforms with secreted, membrane-bound, and intracellular activity (Johnson and Williams, 1993; Minowada *et al.,* 1999, and references therein). A number of genes activated by FGF signaling, such as the T-box family of transcription factors, *caudal*-related genes (*Xcad3*), and a novel TGFβ member (*derriere*) (Smith, 1999; Pownall *et al.,* 1998; Sun, B. I., *et al.,* 1999), are involved in regulating posterior embryonic development. Further complexity is added when negative regulators, such as the *Sprouty* genes in *Drosophila,* chick, and mouse, are involved in the modulation of FGF signaling (Hacohen *et al.,* 1998; Casci *et al.,* 1999; Tefft *et al.,* 1999; Kramer *et al.,* 1999; Reich *et al.,* 1999; Minowada *et al.,* 1999).

The role of FGFs during gastrulation remains obscure and often contradictory due to the reiterative requirement for many family members during this period and the inherent differences in the response of the experimental system. In *Xenopus* gastrula, FGFs have been shown to have mesodermal- and neural-inducing activity while studies of the dominant negative FGFR have revealed a requirement in the development of posterior structures (reviewed by Doniach, 1995). Recently a separable anterior–posterior patterning mechanism has been revealed by studies in Keller explants which have demonstrated a requirement for FGFR signaling in the determination of posterior neural identity (Holowacz and Sokol, 1999). In zebrafish, the formation of the trunk and tail are regulated by FGF activity and T-box family members

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while dominant-negative FGFR studies suggest an indirect involvement of FGF activity in restricting the expression of anterior neural identity (Griffin *et al.*, 1995, 1998; Koshida *et al.*, 1998). Furthermore, anterior development can also be suppressed by exogenous FGF activity in both zebrafish and *Xenopus* (Griffin *et al.*, 1995, 1998; Holowacz and Sokol, 1999 and references therein). In the chick embryo, varying effects have been reported depending on the dosage, the location, and the stage of embryonic development when exogenous FGF is topically applied (reviewed by Kessel and Pera, 1998). These include the induction of anterior and/or posterior neural tissue with or without the formation of mesoderm and, at high doses, the suppression of anterior development (Henrique *et al.*, 1997; Alvarez *et al.*, 1998; Storey *et al.*, 1998).

In the mouse, the effects of FGF signaling have largely been investigated by functionally inactivating various family members. Although several null mutants have not revealed a requirement during gastrulation (Feldman *et al.*, 1995; Mansour *et al.*, 1993; Hèbert *et al.*, 1994), the conditional mutagenesis of *Fgf8* has been successful in determining a role for this family member in the migration of the embryonic mesoderm, formation of the midbrain–hindbrain boundary, and branchial arch development (Meyers *et al.*, 1998; Sun, X., *et al.*, 1999; Trumpp *et al.*, 1999). Furthermore, a requirement for FGF signaling in the correct specification and the migration of the mesoderm during gastrulation and early organogenesis has been inferred from the phenotypes of FGFR1 null mutants and chimeras (Yamaguchi *et al.*, 1994; Deng *et al.*, 1994; Ciruna *et al.*, 1997; Deng *et al.*, 1997).

In this study the role of FGF signaling during mouse development was investigated by examining the effect of delivering exogenous recombinant FGF-4 to the late gastrula embryo. The early response of the embryonic tissues to FGF-4 was assessed by assaying the activity of genes that are expressed in a regionalized manner, such as *T, Hesx1, Otx2,* and *Hoxb1.* After a longer period of culture the effect of ectopic FGF-4 on the developing embryo was determined by assaying gene expression in the forebrain, midbrain, and hindbrain regions and the mesoderm. Recently, Minowada *et al.* (1999) have shown that *Sprouty* gene activity, which apparently represses FGF signaling, is up-regulated in mouse and chick embryos shortly after the application of exogenous FGFs. In the present study, we found that *Spry2* expression was up-regulated shortly after FGF-4 bead implantation, and its activity remained elevated after a prolonged period of *in vitro* development. We propose that the phenotypic consequences of FGF treatment on embryonic development may be due more to a widespread inhibition of FGF signaling activity that accompanies an initial activation by exogenous FGF-4.

MATERIALS AND METHODS

Preparation of FGF-4- and FGF-8b-Coated Beads

FGF protein was delivered to the mouse embryo *in vitro* by the implantation of beads that carry the protein on the heparin-coated surface. Acrylic beads (Sigma) were first rinsed several times in 500 μl of PBS. Approximately 40–50 beads were added to a 5-μl aliquot of recombinant human FGF-4 (Sigma) or recombinant mouse FGF-8b (R&D Systems) solutions diluted in PBS at concentrations 1.0, 0.2, or 0.1 mg/ml. FGF incubation of the beads was done in microdrops, overlaid with light paraffin oil at 4°C for at least 24 h prior to use. The effective concentration of FGF-4 in each soaking solution was determined by ELISA with a goat polyclonal anti-FGF-4 antibody (Santa Cruz Biotechnology Inc.). The ELISA determination showed that approximately 0.3, 0.07, and 0.03 mg/ml effective FGF-4 concentration were found in the three working solutions after the addition of beads. Control beads were incubated in either PBS or BSA (1.0 mg/ml) solutions. Growth-factor-loaded beads were used for up to 3 weeks following the initial preparation.

Embryo Collection, Staging, and Bead Implantation

ARC/s mouse embryos ranging from the early (allantoic) bud to early head fold stages of development (Downs and Davis, 1993) were selected from 7.5 dpc litters. The majority of experiments were performed on embryos with no overt indication of neural plate development. To place the bead in the anterior region of the embryo an incision was made at a point midway between the distal tip and the embryonic–extraembryonic junction on the anterior side. The bead was inserted into the incision using a Leica micromanipulator and was positioned so that it was partially lodged against the interior tissue layers on the anterior side. Manipulated embryos were cultured in medium containing 75% rat serum in Dulbecco's modified Eagle's medium (Sturm and Tam, 1993) without rotation for 2 h to allow healing of the wound which enhanced bead retention. The embryos were cultured in the same medium in a rotating bottle for a further period of 1–3 or 22 h before sampling for analysis.

Analysis of FGF Effects on Morphology and Neural Development

Recombinant FGF-4 and FGF-8b was administered to embryos that ranged from early bud to early head fold stages of embryonic development. From these experiments, we determined that the gastrula embryo was most responsive to the growth factor when it was delivered prior to the initiation of neurulation. FGF-4 was more potent than FGF-8b at all stages examined (data not shown). Further experiments were therefore conducted on the early bud embryo and using FGF-4 only. Embryos implanted with FGF-4 or control beads were removed from culture and examined for morphological effects prior to fixation in 4% paraformaldehyde (PFA) at 4°C. Embryos were then processed for *in situ* hybridization according to the protocol of Wilkinson and Nieto (1993) with minor modifications. Digoxigenin-11–UTP- (Boehringer Mannheim) labeled riboprobes were synthesized using the Ampliscribe kit (Epicentre Technologies). Overnight hybridizations used SDS, 5× SSC, and generally 0.2 μg/μl of probe. No RNase treatment was performed following hybridization while high-stringency posthybridization washes were carried out at 70°C without the use of formamide. Following *in situ* hybridization, embryos were examined and categorized on the basis of the morphology of the head folds, somites, and neural tube. Embryos selected for histology were postfixed in 4% PFA, embedded in paraffin wax, and serially sectioned at 7–8 μm and examined after counterstaining with nuclear fast red.

To assess the immediate impact of recombinant FGF-4 on the developing embryo, the expression of a set of anterior (*Otx2, Hesx1*) and posterior (*T, Hoxb1*) genes was used as early response markers after 3–5 h of culture. To determine the effect of FGF-4 on FGFR activation at both 5 and 24 h after bead implantation, the expression of a negative regulator of FGFR signaling, *Spry2* (Minowada *et al.*, 1999) was also examined. Only the highest dosage of FGF-4 was used for analyzing the immediate effect of FGF-4 on expression. For embryos cultured for 24 h a dose–response analysis was undertaken to determine the threshold effect of FGF activity on morphology. Genes that are expressed in a regional pattern in the early somite stage embryo were studied. These included the panneural marker *Sox2* and markers of the forebrain (*Hesx1, Six3,* and *Fgf8*), the fore-/midbrain region (*Otx2, En1, Wnt1,* and *Fgf8*), the hindbrain (*Krox20, Hoxb1*), and the axial mesoderm and primitive streak (*T, Wnt3a,* and *Fgf8*).

RESULTS

FGF-Treated Embryos Display Dosage-Related Morphological Effects

A dose-dependent response was observed which ranged from severely suppressed anterior development and abnormal axial development to essentially normal morphology (Fig. 1). Several broad morphological criteria were used to categorize the embryo according to the abnormal phenotypes. Embryos were scored for the relative size of head folds, presence of somites, and a visible axis (i.e., a distinct axial midline and neural tube). Embryos from each treatment group were then put into three classes depending on the severity of the morphological disruption (Figs. 1B to 1I, Table 1). The relative proportion of each category correlates with the dose of FGF-4. The wide range of phenotypes may be related to subtle differences in embryonic stage or the amount and activity of growth factor effectively delivered by a bead.

Anterior and Posterior Gene Expression Is Disrupted Shortly after FGF Delivery

The expression of *T* transcript in the treated embryo was examined at either 3 or 5 h after bead implantation to determine whether this T-box family member is responsive to FGF-4. In control embryos *T* expression is restricted to a posterior wedge of germ layer tissue, the ingressing epiblast, and the newly formed mesoderm of the primitive streak (Figs 2A and 2B). Embryos treated with the highest dose of FGF-4 displayed expanded *T* expression that encompasses the epiblast and the mesoderm in the lateral region of the embryo (Figs. 2A and 2C).

In view of the expansion of the *T* expression in the posterior region of the embryo, the expression of *Hesx1* and *Otx2* that characterizes the anterior neural tissue was analyzed. Complementary to the expanded expression domain of *T*, *Hesx1* expression in the anterior endoderm was significantly suppressed (Figs. 2D–2F). In contrast, *Otx2* expression which marks the presumptive fore- and midbrain regions was not altered (Fig. 2G). Similarly, *Hoxb1*, which is expressed in the posterior epiblast and ingressed mesoderm of the primitive streak, did not show any change in expression within 5 h of bead placement (Fig. 2H).

Spry2 Expression Is Up-Regulated after FGF Treatment

The rapid and uniform increase in the posterior expression of *T*, which is known to respond to FGF signaling, suggested that the effect of recombinant FGF-4 may be widespread in the embryo. Previous studies using either MAPK activation assays or DIG-labeled FGF have shown that FGF can diffuse over a considerable distance from either the bead or the transplanted cells that release the growth factor (Labonne and Whitman, 1997; Storey *et al.*, 1998; Christen and Slack, 1999). Studies in *Drosophila* have revealed that the *Sprouty* gene product is a component of a negative feedback loop regulating FGFR and EGFR pathway activity (reviewed by Placzek and Skaer, 1999). Furthermore, *Sprouty* expression can be rapidly induced in response to ectopic FGF in the chick and is involved with FGF signaling in the mouse gastrula, where in the absence of the *Fgf8* activity, no *Sprouty* expression is detected (Minowada *et al.*, 1999). We therefore examined the expression of mouse *Spry2* to determine the extent of FGFR activation following FGF-4 treatment.

Expression of *Spry2* is normally restricted to the primitive streak region of the gastrula embryo (Figs. 3A, 3Bi, and 3C, see Minowada *et al.*, 1999), a region where several FGF genes (*Fgf3, Fgf4, Fgf5, Fgf8,* and *Fgf17*) as well as *Spry4* are expressed. A marked elevation in *Spry2* expression was observed shortly after FGF-4 delivery (Figs. 3A and 3Bii–iv). *Spry2* expression was widespread in the epiblast of the embryo (Fig. 3D), a region which is also a site of *Fgfr1* expression (Yamaguchi *et al.*, 1992). Of particular interest is the absence of *Spry2* expression in the node and head process of the embryo and in extraembryonic tissues (Fig. 3Bii–iv).

To determine whether *Spry2* expression is sustained after FGF treatment, embryos were examined at 24 h of culture. In control embryos, *Spry2* was expressed in sites of endogenous *Fgf* activity such as the region where the caudal midbrain adjoins the rostral hindbrain, the tail bud, and, in some embryos, the rostral part of the forebrain (Fig. 3E; Minowada *et al.*, 1999). *Spry2* was also expressed in the posterior paraxial mesoderm and the tail bud (Fig. 3E). In contrast, FGF-4-treated embryos displayed, besides expression of *Spry2* in the neuroectoderm (Figs. 3F–3H, 3L,and 3M), ectopic expression of *Spry2* in the mesendoderm of the foregut (Fig. 3I) and the midgut (Fig. 3J). In severely affected embryos, histological examination revealed that paraxial mesoderm was absent (Fig. 3J) and *Spry2* mRNA was found in the endoderm lying directly underneath the neural tube (Fig. 3J). The primitive streak of treated embryos was abnormal in tissue organization and failed to express detectable levels of the *Spry2* gene (Fig 3K), although expression

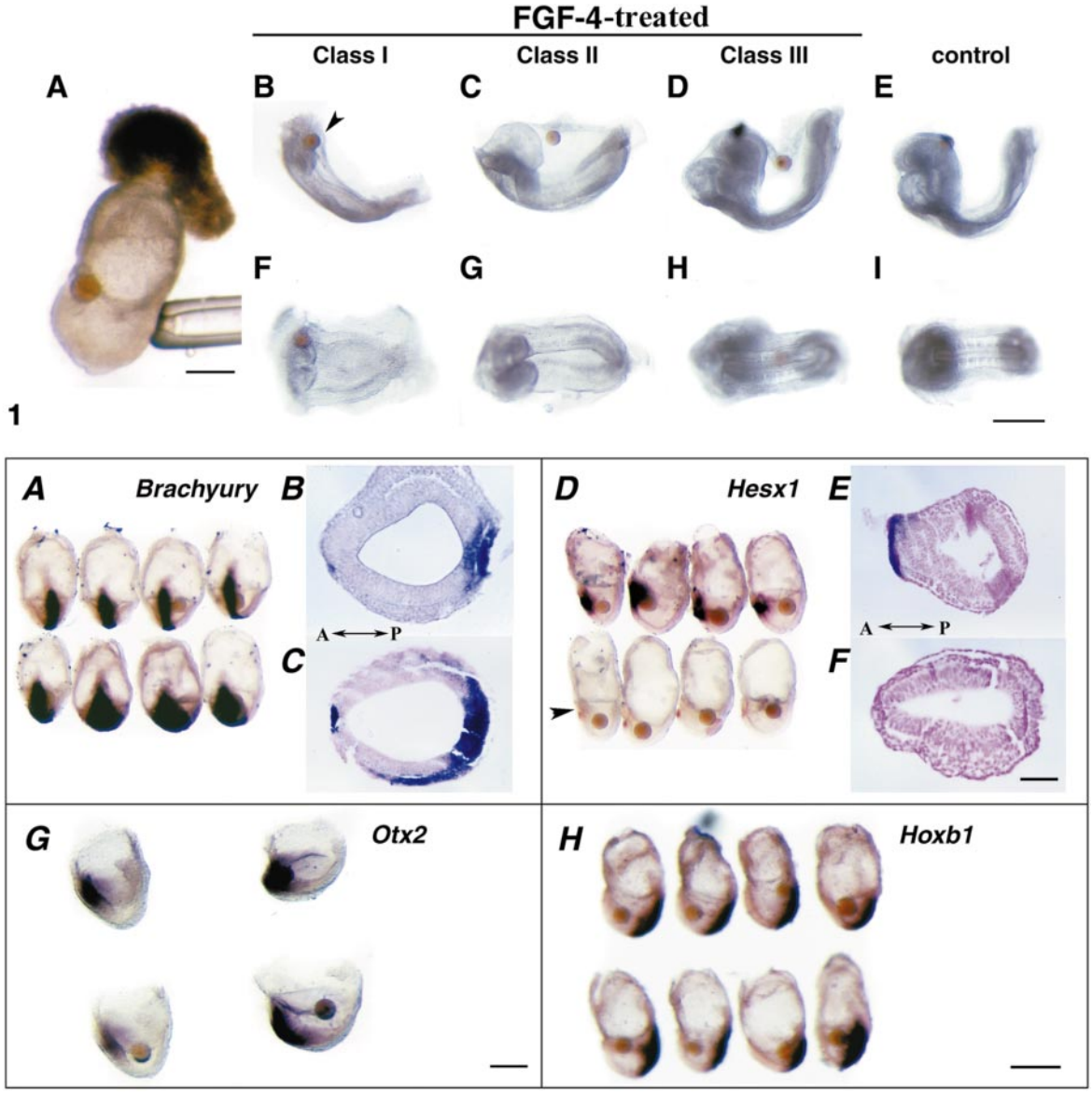


FIG. 1. Bead implantation and the morphological effects of FGF-4. (A) An early-bud-stage mouse gastrula embryo held on the posterior (primitive streak) side by suction to a holding pipette and with a heparin-coated acrylic bead implanted into the anterior region. Bar, 200 μm. (B to E) Lateral, (F and G) dorsal, and (H and I) ventral views of FGF-treated and PBS control embryos. The allantois has been removed. (B, F) Class I embryos showing poor development of the head folds and a foreshortened body axis, absent somites, and no closure of neural tube. (C, G) Class II embryos with moderate head fold development and axis elongation, no morphological delineation of fore- and midbrain segments, and an open neural plate. Only the most anterior somites (that express *Meox1*, data not shown) are formed but are irregularly shaped and loosely organized. (D, H) Class III embryos showing normal head fold with well-delineated primary brain parts, a closed neural tube, and between six and eight pairs of somites. (E, I) Control embryos implanted with PBS-soaked beads. Bar, 150 μm.

FIG. 2. Early response of the embryo to FGF-4 treatment. (A) Expression of *Brachyury* in PBS control (top row) and FGF-4 treated embryo (bottom row) 3 h after implantation of beads. Embryos are viewed from the posterior (primitive streak) side. A uniform expansion of *T* expression in the posterior lateral region of the embryo was observed after 3 h (n = 7/7) and also 5 h (n = 13/13, not shown) when compared to controls (n = 10 each at 3 and 5 h). (B, C) Transverse sections of (B) PBS control embryo showing expression of *T* is normally confined to a posterior wedge of tissue encompassing the ingressing epiblast and newly formed mesoderm and (C) FGF-4-treated embryo showing a broad domain of *T* expression in the posterior epiblast and the mesoderm. (D) PBS control embryos (top row) with normal *Hesx1* expression in the anterior germ layers of the embryo (n = 17, two experiments) and FGF-4-treated embryos (bottom row) 5 h after bead implantation showing either absent (5/15 embryos) or a small focus of *Hesx1* expression (arrowhead, 10/15 embryos). (E, F) Transverse sections of (E) PBS embryo with expression in the anterior endoderm and (F) FGF-4-treated embryo showing absent *Hesx1* expression. Bar, 100 μm. (G) Similar pattern of *Otx2* expression in PBS- (top row, n = 17) and FGF-4-treated embryos (bottom row, n = 17) after 5 h of culture (two experiments). (H) *Hoxb1* expression in PBS (top row, n = 9) and FGF-4-treated embryos (bottom row, n = 9) showing similar expression domains 5 h after treatment. Bar, 200 μm. Double arrows indicate the orientation of the anterior–posterior (A-P) axis of histological sections.

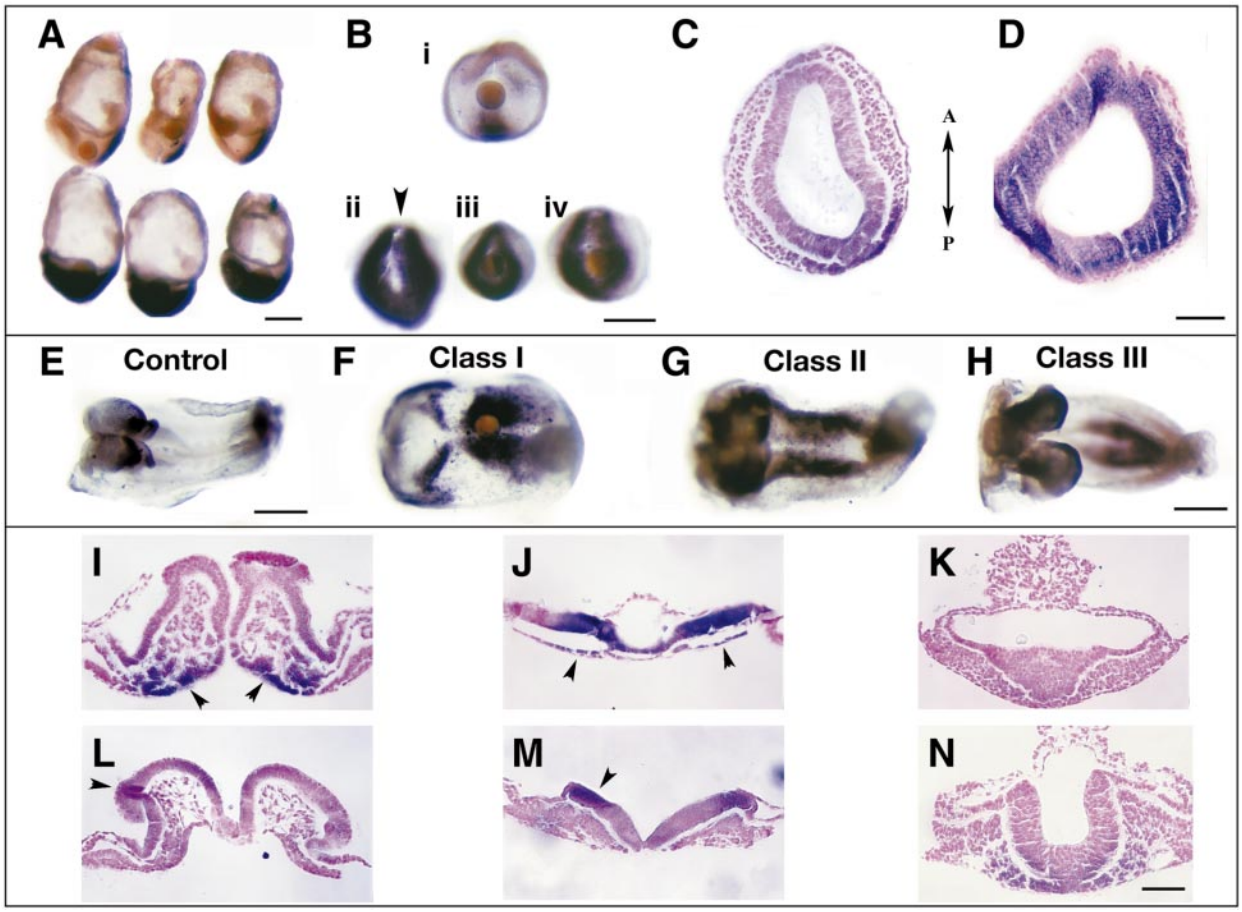


FIG. 3. Ectopic expression of *Spry2* after FGF-4 treatment. (A) *Spry2* is normally expressed in the primitive streak of PBS control (top row, $n = 9$) but is strongly expressed throughout the FGF-4-treated embryos (bottom row, $n = 7$) 3–5 h after treatment. Bar, 200 μm. (B, i) The PBS control embryo viewed from the distal aspect (anterior to the top side) showing *Spry2* expression in the primitive streak and adjacent germ layers on the posterior side. (ii, iii, and iv) Three examples of ectopic *Spry2* activity in FGF-4-treated embryos, showing strong expression throughout the embryo except for the presumptive node and tissues in the anterior midline (arrowhead). Bar, 200 μm. (C, D) Transverse sections of (C) PBS control and (D) FGF-4-treated embryos: *Spry2* expression is normally restricted to the posterior epiblast and nascent mesoderm of the control embryo, but is up-regulated throughout the epiblast following FGF-4 treatment. The shattering of the FGF-4 specimen is due to a sectioning artifact caused by the presence of the heparin-coated bead in this sample. Double arrow indicates orientation of the anterior–posterior (A-P) axis. Bar, 100 μm. (E) Control embryo implanted with PBS bead. *Spry2* expression is normally confined to the midbrain and hindbrain region and the tail bud. Bar, 150 μm. (F–H) After 24 h of *in vitro* development, *Spry2* expression remains strong in the treated embryos: (F) A Class I embryo (12/70 embryos, three experiments), (G) Class II (41/70 embryos), and (H) Class III (17/70 embryos) showing ectopic expression of *Spry2* in the head region and the neural plate in the trunk region. Bar, 100 μm. (I–N) Histological sections of FGF-4-treated embryo showing (I) *Spry2* expression in the mesendoderm (arrowhead) underneath the head folds, (J) expression in the neural plate and the endoderm (arrowheads) in the trunk region that is deficient of paraxial mesoderm, (K) no expression in the posterior neuroectoderm, and (L–M) weak expression throughout the neuroectoderm of the head folds (L, arrowhead) but strong expression in the lateral region of the trunk neural plate (M, arrowhead) and (N) expression in the medial portion of the paraxial mesoderm and the ventral neural tube tissues. Bar, 50 μm.

was observed in the ventral neural tube and the posterior paraxial mesoderm in some embryos (Fig 3N).

Anterior Neural Identity Is Specified but Morphogenesis Is Suppressed in FGF-Treated Embryos

An analysis of the expression of the neural-specific *Sox2* gene revealed that all treated embryos expressed *Sox2* (Figs. 4A–4D) in the neural tube, indicating that neural differentiation has not been impaired. Several regional neural markers were next examined to determine whether FGF-4 treatment has any effect on the segmental delineation of the neural tube. To determine the extent of general anterior and posterior neural development, *Otx2* expression was analyzed in embryos treated with varying amounts of FGF-4 and cultured for 24 h (Figs. 4E–4H). Class I and II embryos

TABLE 1

The Relative Proportion in Three Different Classes of Embryos Treated with FGF-4 by Bead Implantation and Cultured for 24 h *in Vitro*

FGF-4 dosage (mg/ml)[a]					
Working solution	Effective	No. of embryos	Class I[b] (%)	Class II[b] (%)	Class III[b] (%)
PBS control		51	0	4	96
0.1	0.03	45	4	34	62
0.2	0.07	58	9	60	31
1.0	0.3	138	20	68	12

Note. Data are collated from seven experiments.

[a] The dosage of FGF in the working solution is given by weight of recombinant protein per volume and the effective dosage is expressed as the concentration of immunoreactive peptide factor quantified by ELISA.

[b] Examples of the three classes of embryos are shown in Fig. 1 and the morphological criteria for classification are described in the figure legend.

expressed *Otx2* anteriorly although expression was often reduced compared to controls. Two severely affected Class I embryos that lacked head folds still weakly expressed *Otx2* anteriorly (Fig. 4F). In contrast to the uniform suppression of *Hesx1* expression observed soon after bead placement, expression was more variable after a longer period of culture. Expression was often reduced to a small anterior focus in Class I and II embryos although some Class II embryos strongly expressed *Hesx1* throughout the rudimentary head folds (Figs. 5A–5D). The remaining Class III embryos exhibited normal *Hesx1* expression. A similar reduction in *Six3* expression was also observed in Class I and II embryos while Class III embryos showed *Six3* expression similar to controls (Figs. 5E–5H). *Fgf8* is expressed in the anterior neural ridge of the forebrain later than *Hesx1* and *Six3*. Of all the forebrain markers assessed, *Fgf8* appeared to be most sensitive to the dosage effects of FGF-4. All classes of FGF-treated embryos failed to express detectable levels of *Fgf8* in the region corresponding to the presumptive forebrain (Figs. 5I–5L). *Hoxb1* was expressed in the posterior regions of most treated embryos, except for two Class I embryos with severely reduced head folds where gene expression seemed to extend more rostrally (Figs. 4I–4L). Considered collectively, these results suggest that the development of the anterior neural tube, specifically the forebrain, was adversely affected in the FGF-4-treated embryos.

Aberrant Development of the Midbrain–Hindbrain Region, Primitive Streak, and Paraxial Mesoderm

In the early-somite-stage embryo, *Fgf8* is expressed in the midbrain–hindbrain boundary in addition to the anterior neural ridge of the forebrain (Fig. 5I). In the FGF-4-treated Class I and Class II embryos diffuse *Fgf8* expression was detected in the ventral tissues in the head folds (Figs. 5J and 5K). Histological examination revealed that *Fgf8* expression was largely confined to the mesoderm and endoderm underlying the head fold and around the foregut portal (data not shown). A weak expression of *Fgf8* was detected in the presumptive midbrain to hindbrain region of the Class III embryos (Fig. 5L). An analysis of other genes such as *En1* and *Wnt1* that are expressed in the midbrain revealed that these genes were expressed in a more rostral domain in the neural tube of the FGF-4 treated Class II embryos (*En1*, Figs. 6A–6D; *Wnt1*, Figs. 6E–6H). This anterior displacement of expression might be related to the lack of forebrain differentiation. Furthermore, the expression of *Wnt3a* in the presumptive midbrain was often absent (Fig. 6J) or markedly reduced in treated embryos (Figs. 6K). *Krox20*, which was expressed in the third and fifth rhombomeres of the hindbrain of the control embryos (Fig. 6M) was expressed in some Class I (Fig. 6N) and Class II embryos. In some embryos, expression was compressed (Fig. 6O) while in others rhombomeric separation was lost. The Class III embryos expressed a normal pattern of *Krox20* activity (Fig. 6P). FGF-4 treatment therefore may also adversely affect the patterning of the midbrain and the hindbrain.

In view of the expanded expression of *T* activity soon after FGF-4 treatment, the expression of *T*, *Wnt3a*, and *Fgf8* in the primitive streak (Figs. 5I, 6I, 7A, and 7F) was studied in embryos cultured for 24 h to assess the impact of growth factor treatment on the formation of the mesoderm. Consistently, an expanded expression pattern of all three genes was found in the FGF-4-treated Class I and Class II embryos (Figs. 5J, 5K, 6J, 6K, 7B, and 7C). In the Class III-treated embryo, the *T* gene was more strongly expressed (Fig. 7D) but the expression of the *Fgf8* and *Wnt3a* genes was comparable to the controls (Figs. 5L and 6L). Histological examination of the FGF-4-treated embryo showed that there was an accumulation of cells in the primitive streak (Figs. 3K and 7H). Furthermore, the treated embryos contained an excess amount of posterior paraxial and tail bud mesoderm (Figs. 3K and 3N) but were markedly deficient in trunk paraxial mesoderm (Figs. 3J and 7G). Most FGF-4-treated embryos developed a notochord-like axial structure

with a *T*-expressing midline which appeared broader and more diffuse than controls (compare Figs. 7A with 7B–7D). In some Class I embryos that were deficient in paraxial mesoderm, the *T* gene, like the *Spry2* gene, was expressed in the endoderm underlying the neural plate (Figs. 3J and 7G).

DISCUSSION

FGF-4 Activity Suppresses the Development of the Anterior Neural Tube

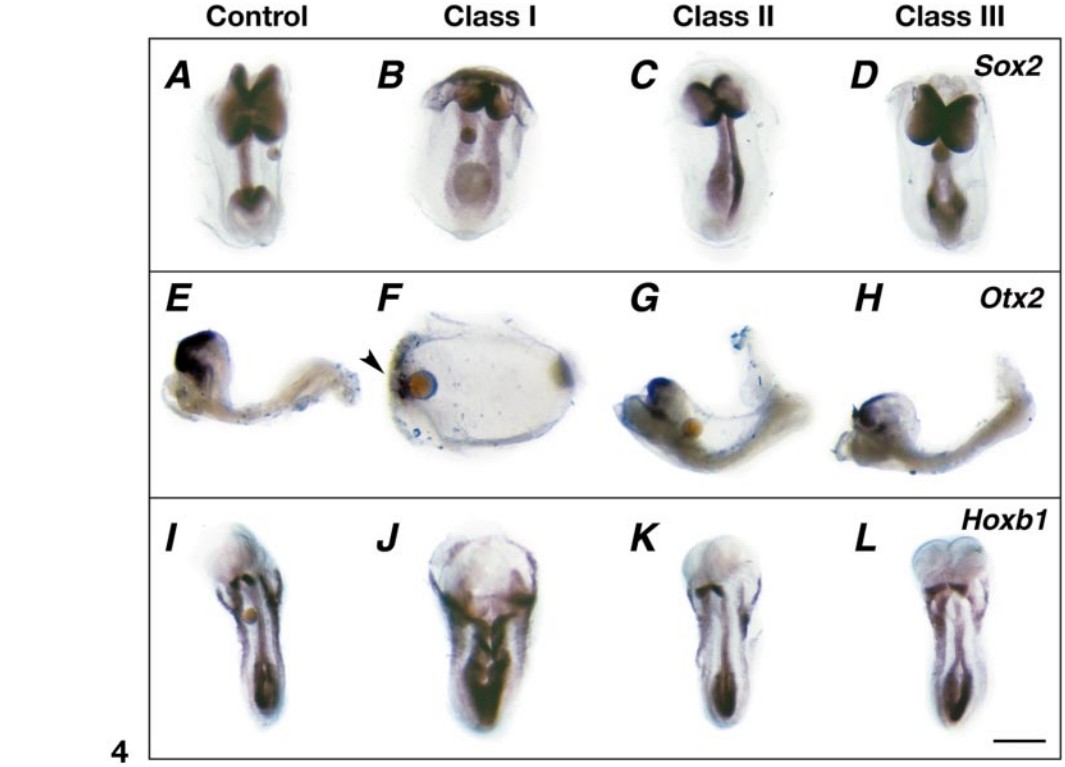
The results of our study show that the disruption of the FGF signaling process may suppress anterior neural development in the mouse embryo, as in the embryos of *Xenopus*, zebrafish, and chick (Isaacs *et al.*, 1994; Griffin *et al.*, 1995; Woo and Fraser, 1997; Henrique *et al.*, 1997; Lombardo and Slack, 1998; Holowacz and Sokol, 1999). When the development of the neural tube is assessed by the expression of region-specific genes, it is revealed that FGF-4 treatment leads mainly to a defective formation of the forebrain and the midbrain. *Hesx1* expression was reduced shortly after FGF-4 treatment. This early decrease in *Hesx1* expression may subsequently impact on the expression of other forebrain genes such as *Six3* and *Fgf8* resulting in the observed suppression of forebrain growth. Furthermore, the absent or the anterior shift in the expression of several genes expressed in the midbrain and hindbrain regions in FGF-treated embryos could also be related to this initial reduction of *Hesx1* expression.

It has been suggested that in the chick embryo, neural development progresses through three distinct phases: competence, induction, and regionalization (Streit *et al.*, 1997). The late gastrula mouse embryo appears to be restricted in its competence to respond to neural-inducing signals as determined by organizer transplantation assays (Beddington, 1994; Tam *et al.*, 1997) and anterior ectoderm may have been determined for a neural fate at this stage (Ang and Rossant, 1993). Ectopically administered FGF-4 therefore may alter only regionalized neural development rather than neural induction per se. In *Xenopus*, FGF activity has been shown to be sufficient to induce ectopic *Krox20* expression in animal cap explants that contain neural progenitors and dorsal mesoderm (Cox and Hemmati-Brivanlou, 1995). However, in zebrafish, the induction of hindbrain-specific gene activity cannot be achieved by FGF activity alone but requires an interaction with the marginal zone of the blastoderm (Woo and Fraser, 1997). In the chick, FGF activity is sufficient to organize an ectopic midbrain–hindbrain junction and induce *En1* expression (Crossley *et al.*, 1996; Martinez *et al.*, 1999; Shamin *et al.*, 1999). It must be noted that in our study, there is no compelling evidence that FGF-4 activity leads to the induction of ectopic neural tissue. This could reflect a temporal divergence or varying sensitivity of gene activation during the formation of the midbrain–hindbrain junction (Reifers *et al.*, 1998; Lun and Brand, 1998; Shamin *et al.*, 1999) and a restricted competence of the late mouse gastrula to respond to exogenous inductive signals.

Does Exogenous FGF-4 Affect Posterior Development?

Although a positive regulatory relationship involving FGF signaling and *Brachyury* transcriptional activity has been shown in both *Xenopus* (Isaacs *et al.*, 1994; Latinkic *et al.*, 1997; Casey *et al.*, 1998) and zebrafish (Griffin *et al.*, 1995, 1998), evidence supporting a similar relationship in the mouse embryo is less well established (see Schmidt *et al.*, 1997). The immediate up-regulation of *T* expression soon after FGF-4 treatment indicates that *T* transcription is responsive to exogenous FGF-4 in the mouse embryo. Mutation in *T* results in a homozygous lethal phenotype due to a deficiency of posterior embryonic tissue whereas heterozygotes exhibit a milder phenotype suggesting sensitivity to the levels of T-box protein activity (Smith, 1999). In this study, the broad *T* expression observed soon after FGF-4 treatment may reflect an expansion of the posterior tissue domain of the embryo. When considered in conjunction with the known posteriorizing activity of FGF and the ability of *T*-expressing tissues to suppress anterior neural differentiation (Ang *et al.*, 1994; Cox and Hemmati-Brivanlou, 1995; Woo and Fraser, 1997; Koshida *et al.*, 1998), the effects of FGF-4 on anterior development could be mediated by a posterior expansion of a *T*-dependent effect. However, it is probably more likely that ectopic FGF activity directly suppresses anterior neural identity as has

FIG. 4. FGF-treated embryos showing reduced anterior neural gene expression. (A) PBS control ($n = 8$), (B) Class I ($n = 6$), (C) Class II ($n = 18$), and (D) Class III ($n = 12$) embryos all expressed *Sox2* activity. (E) Control PBS embryos with normal morphology and *Otx2* expression in presumptive forebrain and midbrain ($n = 5$). (F) A maximally affected Class I embryo with a small anterior focus of *Otx2* expression. Other Class I embryos show reduced expression domain ($n = 14$). (G) Class II ($n = 10$) and (H) Class III ($n = 8$) embryos showing normal *Otx2* expression in anterior neuroectoderm. (I) PBS control embryo with *Hoxb1* expression in rhombomere 4 and in the posterior neural tube and mesoderm. (J) A Class I embryos showing an expanded *Hoxb1*domain of in the posterior tissues. Other Class I embryos ($n = 6$, not shown) show a posterior expression pattern similar to controls but expression in the rhombomere is absent in one embryo and in the others is confined to the lateral edges of the open neural plate. (K) Class II ($n = 13$) and (L) Class III ($n = 12$) embryos displaying normal *Hoxb1* expression in rhombomere 4 and posterior neural tube. Bar, 150 μm.



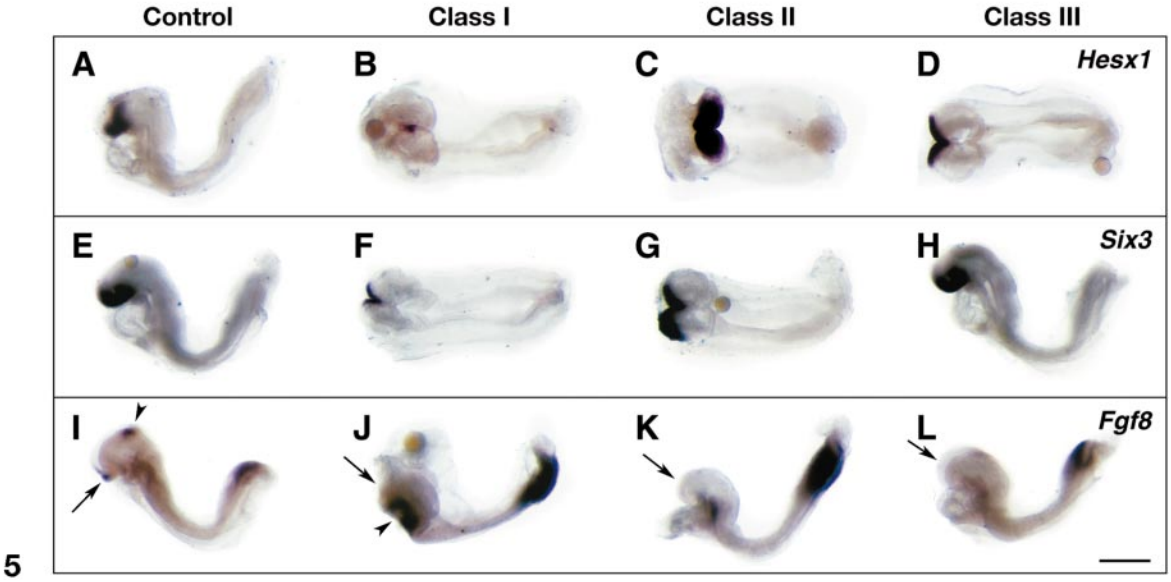
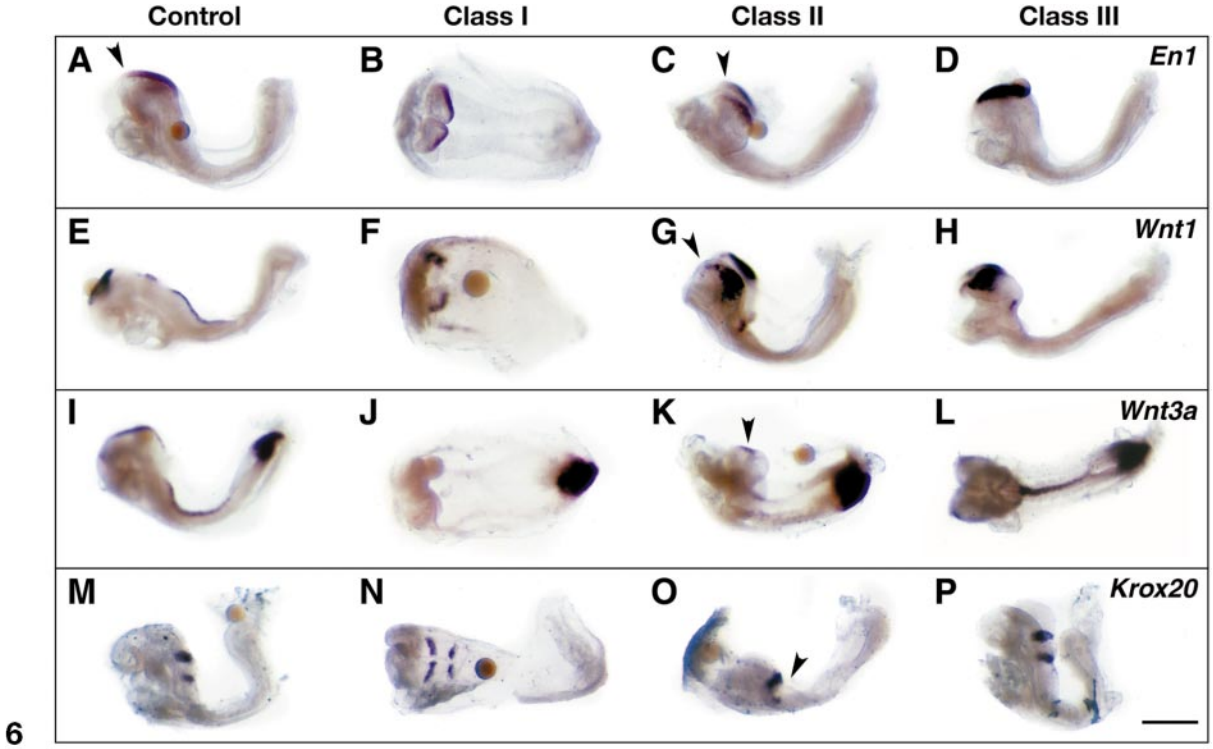


FIG. 5. Forebrain development is suppressed by FGF-4 treatment. (A–D) *Hesx1* expression. (A) Control embryo shows normal expression in the forebrain tissues ($n = 9$). (B) Class I embryos ($n = 4$) displayed a reduced focus of *Hesx1* expression. (C) Some Class II embryos ($n = 6$) had a similar reduction in *Hesx1* activity but 3 embryos (as shown in the figure) expressed *Hesx1* strongly in the entire head fold. The remaining Class II ($n = 7$) and (D) Class III embryos ($n = 2$) show *Hesx1* activity similar to the controls. (E–H) *Six3* expression. (E) Control embryo. (F) Class I embryo shows a markedly reduced *Six3* expression, while (G, H) the Class II and all Class III embryos show a normal expression pattern. (I–L) *Fgf8* expression. (I) The expression of *Fgf8* in the anterior neural ridge of the forebrain (arrow), the mid- and hindbrain junction (arrowhead) and the primitive streak of control embryo ($n = 9$). (J, K, L) Expression of *Fgf8* in the presumptive forebrain region (arrows) is absent in the 26 of 27 treated embryos (Class I $n = 3$, Class II $n = 14$, Class III $n = 10$). In (J) the Class I embryo, *Fgf8*, is expressed in the mesendoderm of the foregut and not in the neuroectoderm. An expanded posterior expression domain is also apparent in the tail bud/primitive streak region of Class I and II embryos. Bar, 150 μm.



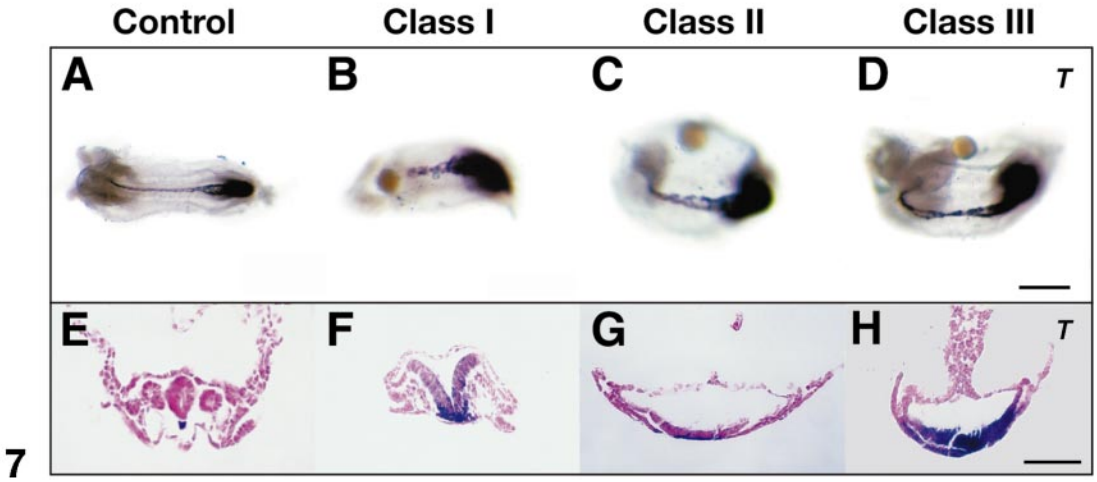


FIG. 6. Midbrain and hindbrain gene activity is disrupted by FGF-4 treatment. (A) Normal expression of *En1* in the caudal midbrain and rostral hindbrain neuroectoderm. The anterior border of the expression domain (arrowhead) does not extend into the forebrain. (B) A Class I embryo which showed weak *En1* expression although four others failed to initiate any expression. (C) Class II embryos ($n = 16$) expressed *En1* more rostrally in the neural tube (arrowhead), consistent with the loss of forebrain tissues. (D) Normal expression in Class III embryos ($n = 8$). (E–H) *Wnt1* expression. (E) Normal expression of *Wnt1* marks the caudal midbrain region and dorsal neural tube in the control embryo. (F) Anterior expression of *Wnt1* was present in most Class I embryos (8/10 embryos). In 3 embryos, expression extended caudally into the lateral regions of the open neural plate (not shown). (G) A Class II embryo ($n = 17$) expressing *Wnt1* more rostrally in the small head folds (arrowhead) ($n = 8$). (H) Class III embryos ($n = 9$) showing normal expression. (I–L) *Wnt3a* expression. (I) Expression in the dorsal/lateral midbrain region, dorsal neural tube, and primitive streak/tail bud of the control embryo. (J) Class I embryos ($n = 6$) showing no *Wnt3a* expression in the anterior neuroectoderm. (K) Class II embryos ($n = 15$) which developed rudimentary head folds had detectable expression (arrowhead) along the lateral regions of the open neural plate. (L) A Class III embryo showing normal *Wnt3a* expression. (M) Formation of the hindbrain rhombomeres 3 and 5 was revealed by *Krox20* expression. (N) Normal *Krox20* expression was observed in only one Class I embryo (shown) while in others expression was reduced to one band or absent. (O) Only one stripe of *Krox20* expression is observed in this Class II embryo (arrowhead) while (P) the Class III embryo displays normal expression. Bar, 150 μm.

been shown in FGF-treated Keller explants (Holowacz and Sokol, 1999).

Effects of FGF-4 on Embryonic Development Is Mediated by the Negative Regulation of FGF Signaling

Interestingly, viral overexpression of *Sprouty* in chick limb buds causes a phenotype consistent with a reduction in FGF signaling. However, the data also raise the possibility that in some cases *Sprouty* gene overexpression may enhance FGFR signaling (Minowada *et al.*, 1999). In this study, the widespread ectopic expression of *Spry2* soon after FGF-4 treatment in the tissues that are also known to display FGFR1 activity implies that FGFR signaling is widely activated in the embryo. The activation of FGFR signaling is apparently also not restricted to the tissues in the vicinity of the exogenous source of FGF-4. This observation is in marked contrast to the findings in chick and *Xenopus* embryos where a more local effect of FGF has been shown after bead or cell implantation (Storey *et al.*, 1998; Christen and Slack, 1999). It is important to consider the differences between these experimental models that might account for the more widespread effect of FGF-4 in the mouse gastrula. The mouse gastrula is much smaller by comparison with the chick and *Xenopus* gastrula, and as such, a soluble growth factor placed at a specific site may reach more embryonic tissues. The presence of an enclosed amniotic cavity may also facilitate the trapping and indiscriminate transfer of the growth factor released from the implanted bead to other regions of the embryo.

The initial activation of a negative FGF signaling regulator and its subsequent ectopic expression therefore complicate the interpretation of experiments of this kind. Is the observed phenotype a result of activation or inactivation of the signaling pathway? Although the variable phenotypes reported for FGFR1 null mutants and chimeras suggest multiple requirements for FGFR1 signaling during early development, there are several parallels that can be drawn with the phenotypes observed in this study. Like FGF-4-treated embryos, FGFR1 null mutants develop with small disorganized head folds and a disruption in the formation of the trunk mesoderm (Yamaguchi *et al.*, 1994; Deng *et al.*, 1994). However, in contrast, *Fgfr1*$^{-/-}$ cells in the chimera fail to differentiate into mesoderm and adopt a neural fate, a phenotype not observed after FGF-4 treatment (Ciruna *et al.*, 1997; Deng *et al.*, 1997). In the most severely affected Class I embryos a dramatic decrease in the amount of paraxial mesoderm and an accumulation of cells in the posterior mesoderm and the primitive streak were observed. Less severely affected Class II embryos showed a disruption in somitogenesis in the trunk suggesting that the segmentation process had stalled. However, the formation of the notochord appeared largely unaffected in the majority of embryos although instances of broader *T*-expressing midline tissues were observed in some Class I embryos. The initial expansion of *T* expression could also have impacted on the differentiation of the ingressing mesoderm, a region of high endogenous FGF activity. As there appears to be extensive ectopic *Spry2* expression following FGF treatment it is likely that endogenous FGFR signaling has been suppressed or blocked (Minowada *et al.*, 1999). This may affect the normal morphogenetic signals provided by the FGF signaling pathway that are required for mesoderm formation. The widespread expression of a negative regulator (*Spry2*) suggests that these morphogenetic effects may be a consequence of a suppressive activity counteracting the overactivation of the FGFR signaling pathway by excessive FGF.

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Note added in proof. Further evidence that exogenous FCF's can ectopically induce *Spry2* expression has been reported in the chick embryo.

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FIG. 7. (A–H) Notochord differentiation following FGF-4 treatment. (A) Whole mount and (E and F) histological section of control embryos showing the expression of *T* in the in the notochord (A, E), the primitive streak (A, F), the nascent mesoderm (F), and the tail bud (A). (B) Whole mount and (G, H) histological sections of Class I embryos showing broad *T* expression in the foreshortened body (B) and *T* expression in the (G) endoderm underlying the open neural plate and (H) the posterior tissues. (C) Class II and (D) Class III embryos showing *T* expression in axial mesodermal tissues, which are often less compact than the controls. Bar, 150 μm (AD) and 100 μm (E–H).

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